Effects of muCT radiation on tissue engineered bone-like constructs

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Abstract

High-resolution, non-destructive imaging with micro-computed tomography (μCT) enables *in situ* monitoring of tissue engineered bone constructs. However, it remains controversial, if the locally imposed X-ray dose affects bone development and thus may influence the results. Here, we developed a model system for μCT monitoring of tissue engineered bone-like constructs. We examined the *in vitro* effects of high-resolution μCT imaging on the cellular level by using pre-osteoblastic MC3T3-E1 cells embedded into three-dimensional (3D) collagen type I matrices. We found no significantly reduced cell survival 2 h after irradiation with a dose of 1.9 Gy. However, 24 h post-irradiation, cell survival was significantly decreased by 15 % compared to non-irradiated samples. The highest dose of 7.6 Gy decreased survival of the pre-osteoblastic MC3T3-E1 cells by around 40 % at 2 d post-irradiation. No significant increase of Alkaline Phosphatase *(*ALP) activity at 2 d post-irradiation was found with a dose of 1.9 Gy. However ALP-activity was significantly decreased after 7 d. Using our model system, the results indicate that μCT imaging with doses as low as 1.9 Gy, which is required to obtain a reasonable image quality, can induce irreparable damages on the cellular level.

Keywords: *Alkaline Phosphatase Activity (ALP); Cell survival; Collagen; MC3T3-E1 pre-osteoblastic cells; Micro-computed tomography (μCT); Tissue Engineering*

Introduction

The *in vitro* engineering of functional tissue constructs gained importance in the last decade as the shortage of organs and tissues for transplantation increased [21], but also for safety and efficacy testing of new therapeutics [8,12,15]. So far, quality testing of engineered tissue constructs relied on destructive techniques including reverse transcription polymerase chain reaction (RT-PCR) to analyze gene expression or histology to assess organization and integrity of the tissue. Thus, monitoring over time and functional optimization in response to assessed changes in tissue morphology and functionality was not possible with these techniques. Other methods, such as magnetic resonance imaging (MRI) or positron emission tomography (PET) do not provide sufficient spatial resolution [10,17].

μCT was shown to be useful as a non-destructive method in imaging 3D bone microarchitecture at resolutions in the order of 10 μm in relatively short acquisition time (5-30 min), and at low cost [2,3,19]. In recent studies, μCT was used to monitor the development of 3D *in vitro* engineered bone-like tissues [4,13,14], cartilage-like tissues [23], or skin-like layers [20] over time. However, a draw-back of obtaining high-quality μCT images may be the locally deposed radiation dose and its potential effect on cell survival, cell functionality and cell development [11].

Several studies have examined the effects of ionizing radiation on bone cells in a two-dimensional (2D) environment at doses up to 8 Gy [5,6,9]. While the proliferation of MC3T3-E1 pre-osteoblastic cells and osteoblast-like cells from the rat calvarium was not affected by a radiation dose of 0.4 Gy, a dose of 4.0 Gy significantly decreased proliferation in all studies, in some cases significantly altered ALP activity, collagen production and vascular endothelial growth factor (VEGF) secretion as compared to non-irradiated control samples. However,

irradiation effects on 3D tissue engineered constructs, which mimic native 3D tissue and organ structures better than 2D environments [8,12], are not fully understood. Specifically, the critical dose, which can maximally be deposited onto the constructs without inducing cellular damages, is not clear. Since there is a big difference between a flat layer of cells and a complex 3D tissue [1] it is important to investigate the irradiation effect in 3D tissues.

The goal of this study was to assess this maximum dose. We have developed a model system for μCT monitoring of 3D tissue engineered constructs, and to elucidate effects of the ionizing radiation applied by μCT imaging on the cellular level by using MC3T3-E1 pre-osteoblastic cells embedded in 3D collagen type I matrices mimicking bone-like tissue [7,16]. We hypothesized that the locally deposed X-ray dose by high-resolution μCT imaging may cause alterations on the cellular level in a dose- and time-dependent manner. This may affect the development of engineered 3D tissue constructs for clinical applications, but also the results of pharmaceutical studies for testing new therapeutics.

Materials & Methods

Dose measurement with thermo luminescent detectors

The radiation dose occurring with typical settings for μCT scanning was measured with thermo luminescent detectors (TLD) made of lithium fluoride powder embedded in a glass tube of 12 mm length and 1.4 mm diameter (TLD-700, Harshaw/Filtrol, Solon, OH, USA). For the μCT measurement, the TLD's were placed in a cylindrical polymethyl methacrylate (PMMA) cylinder (inner diameter: 2 mm, outer diameter: 8 mm) to ensure corresponding radiation doses as applied to the cells embedded into the collagen matrix. The μCT (μCT40, Scanco Medical AG, Brüttisellen, Switzerland) was operated at a peak voltage of 50 kV with an integration time of 100 ms in all possible scanning modes. The three primary scanning modes provided by the microCT manufacturer were "standard resolution" mode (250 projections/180°, 1024 samples per projection), "medium resolution" mode (500 projections/180°, 1024 samples), and "high resolution" mode (1000 projections/180°, 2048 samples). In each mode, five different nominal resolutions were available namely 12, 16, 20, 30, and 36 μm for the standard and medium resolution mode, and 6, 8, 10, 15, and 18 μm for the high resolution mode. These nominal resolution values correspond to the settings that can be chosen to run the scans and do not represent the actual spatial resolution, which is typically lower. The best spatial resolution (10% MTF) at 6 μm voxel size is specified by the manufacturer to be 10 μm. After irradiation, the TLD's were read out with a photo-multiplier using a standard heating protocol.

For all cell experiments the medium resolution mode at 20 μm nominal resolution was used, but with a threefold increased integration time resulting in a calculated base dose of 1.9 Gy per measurement. To achieve the higher doses of 3.8, 5.7, and 7.6 Gy, the base measurement was repeated 2, 3, and 4 times, respectively. The total times for application of these radiation doses were 1.5 h, 3 h, 4.5 h and 6 h, respectively. To examine potential differences in image quality, a

bovine trabecular bone sample was scanned with μCT in four different modes: in standard resolution mode at 30 μm and 16 μm nominal resolution, as well as in high resolution mode at 15 μm and 8 μm nominal resolution.

Culture of pre-osteoblastic MC3T3-E1 cells

A MC3T3-E1 subclone 24 (LGC Promochem Sarl, Molsheim Cedex, France) was cultured between passage 6-10 in alpha-modified minimum essential medium containing 10 % fetal bovine serum (FBS, both Invitrogen, Basel, Switzerland), 1 μg/mL bone morphogenic protein-2 (BMP-2) produced as previously shown [22], and 25 mMol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich, Buchs, Switzerland).

Preparation of 3D collagen matrices

Collagen type I matrices (Vitrogen 100, Cohesion, Palo Alto, CA, USA) of 2 mg/mL were used. Matrix formation was initiated by raising the pH to physiological conditions (pH 7.4) by adding 0.1 M NaOH to the collagen solution. To ensure homogenous cell encapsulation, the matrices were pre-solidified at 37° C for 5 min prior to the addition of 500'000 cells/mL collagen I. Matrices with a volume of 30 μL were then filled into sterilized (70% Ethanol, exposure to UV light) PMMA sample holders with a capacity of 35 μL and incubated for 30 min at 37° C to solidify. The sample holders containing the 3D cell cultures were then kept in 1 mL medium in a well of a 24-well plate under standard cell culture conditions.

Irradiation with μCT

A high-resolution micro-computed tomography system (μCT40, Scanco Medical AG, Brüttisellen, Switzerland) was used to apply the ionizing radiation to the cells embedded in the

3D collagen type I matrices. The system was operated in all cell experiments at a peak voltage of 50 kV with an integration time of 300 ms at a nominal resolution of 20 μm. The absolute dose delivered to the samples was assessed in an independent experiment using TLD's. In all experiments, cultured MC3T3-E1 cells embedded in 3D collagen type I matrices were exposed to radiation 24 h after seeding into the collagen matrix. For cell survival experiments, cells were irradiated with doses of 1.9, 3.8, 5.7, and 7.6 Gy. For DNA and ALP assays, a dose of 1.9 Gy was applied. Control samples were placed inside the μCT, shielded with a lead cylinder against potential irradiation. Temperature effects on cell survival and ALP expression during a μCT run can thus be excluded. Survival experiments as well as ALP/DNA experiments were all compared with this μCT control.

Cell survival after μCT irradiation

Cell survival after μCT irradiation was determined with a Live/Dead cytotoxicity kit (Molecular Probes, Eugene, OR, USA). This assay was performed 2 h and 24 h post-irradiation (per dose, and per timepoint: $n_{Samples} = 3$; $n_{Control} = 9$). The samples were washed in phosphate-buffered saline (PBS, Sigma Aldrich, Basel, Switzerland) and stained for 45 min with 200 μL Live/Dead staining solution (4 μM ethidium homodimer-1, 2 μM calceinAM in PBS). The samples were then washed in PBS and fixed in 4 % glutaraldehyde. Epifluorescence images were taken at five randomly selected locations (area of ~2 x 1 mm^2) within the matrix by using a fluorescence microscope (Zeiss Axiovert 135, Feldbach, Switzerland) and the fluorescence for calceinAM was determined with an excitation wave length of 495 nm and an emission wavelength of 515 nm. For ethidium homodimer-1, an excitation wavelength of 495 nm and an emission wavelength of 635 nm were used, respectively. The number of surviving cells per total number of cells (live cells plus dead cells, between 30 and 100 cells in total, per location) was

determined by manual counting. The cell numbers of the irradiated samples were normalized to the non-irradiated control samples, which were placed into the μCT with lead shielding.

ALP activity assay

Each sample was homogenized in 300 μL lysis solution (0.2 % v/v Triton X-100 in 0.56 M 2-amino-methyl -1-propanol pH 10.0). Total number of samples/controls per timepoint: $n_{Samples}$ = 10; $n_{Control}$ = 10. From each sample an aliquot was used to determine ALP-activity. Briefly, 150 μL of the lysed cell suspension was mixed with 20 μL of P-nitrophenyl phosphate solution (74 mg/mL in 0.56 M 2-amino-methyl-1-propanol pH 10.0) and incubated at 37° C for 30 min. The reaction was stopped by addition of 85 μL 2 N NaOH. The ALP activity was then determined by measuring the absorbance at 405 nm with a spectrometer (Spectronic Genesys 2PC, Thermo Fisher Scientific, Milford, MA, USA).

DNA determination assay

To determine the DNA content per gel, 30 μL of the lysed cell suspension was neutralized by addition of 270 μL TE-buffer (100 mM TrisHCl pH 7.6, 1 mM EDTA) and centrifuged for 15 min at 14000 rpm. 270 μL of sample or DNA standard was then mixed with 270 μL Pico Green (Molecular Probes, Eugene, OR, USA) that was diluted 1:200 in TE-buffer. Total number of samples/controls per timepoint: $n_{Samples}$ = 10; $n_{Control}$ = 10. Fluorescence was measured in triplicates using a spectrometer (Synergy HT, BioTek Instruments, Inc, Winooski, VT, USA) at 520 nm (excitation wavelength: 480 nm).

Statistical analysis

The cell survival experiments were performed at different doses, and are shown as mean values ± standard deviation Total number of samples: per dose and per timepoint: $n_{Samples} = 3$; $n_{Control} = 9$). Each sample point was normalized by its corresponding control sample. Comparative analysis was performed with a two-way ANOVA. To test for significant differences between the dose groups, a pair-wise t-test was applied with Holm correction for multiple testing, for both the 2 h and 24 h time groups. To examine significant differences between the 2 h and 24 h time groups within a dose group, we used a Student's t-test. Significant differences were accepted for $p < 0.05$.

The results of the DNA and ALP tests were statistically analyzed using a pair-wise Wilcoxon rank sum-test with Holm correction for multiple testing. Total number of samples/controls per timepoint: $n_{Samples} = 10$; $n_{Control} = 10$. Differences between two data sets were considered statistically significant for $p < 0.05$. For all statistical analysis we used "R", a language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).

Results

Dose measurement and image quality

To investigate the relation between applied dose and image quality, a bovine trabecular bone sample was scanned with µCT applying the doses 0.150 Gy and 0.528 Gy in the Standard resolution mode, and 0.458 Gy and 1.980 Gy in the High resolution mode (Fig. 1A-C). Better image quality (higher resolution) resulted from higher applied doses (Fig. 1A). The grey-level images show a better quality by increasing the resolution from 30 to 8 µm, and with changing the imaging mode from Standard to High resolution. In addition, the binary images in High resolution mode were found to better display the "real" trabecular structure as compared to the binary images in the Standard resolution mode. However, the differences are relatively small and depending on the application a low resolution mode may yield reasonable results at an acceptable dose. We show, that the relation between dose and image quality, here represented as nominal resolution, can be modeled as a power law with exponents 1.74, 1.76, and 2.03, for the Standard, Medium and High resolution mode, respectively (Fig. 1B,C).

Cell survival after µCT imaging is dose and time-dependent

To examine the effects of high-resolution µCT imaging on a cellular-level, we first evaluated the survival rate 2 h and 24 h post-irradiation, with doses of 1.9, 3.8, 5.7 and 7.6 Gy, respectively, using custom-made PMMA sample holders. We found no significant increase in cell death for a dose of 1.9 Gy immediately (2 h) after irradiation as compared to the non-irradiated control group. However, 24 h post-irradiation, cell survival was significantly decreased by 15 % over the control group. Doses after exposure to 3.8 Gy and 5.7 Gy resulted in survival rates of around 80 %. While we did not find a significant decrease in cell survival after exposure to 3.8 Gy between 2 h and 24 h post-irradiation, we demonstrate a significant decrease after exposure to

5.7 Gy by 15 %. The highest dose of 7.6 Gy decreased the survival rate of the cells by around 40 % 24 h post-irradiation as compared to non-irradiated control samples. Nevertheless, no significant difference between 2 h and 24 h post-irradiation was observed for this highest dose.

Time-dependent decrease of ALP-activity per cell

To further investigate the cellular effects of high-resolution μCT imaging, we applied a dose of 1.9 Gy and analyzed ALP activity. When ALP-activity was normalized with the DNA content of the same sample, we found no significant increase in ALP-activity 2 d post-irradiation, but a significant decrease to about 60 % at 7 d (Fig. 3).

Discussion

This is one of the first studies investigating the effect of ionizing radiation of high-resolution μCT measurements on a cellular level by using a 3D tissue-like model system. Using our model system, the results indicate that μCT imaging with doses as low as 1.9 Gy, which is required to obtain a reasonable image quality, may induce irreparable damages on the cellular level.

The dose applied by a μCT measurement depends on the imaging mode and scanner settings, which are adapted to the features of interest. For instance, if bone volume density is of interest, a relatively low image quality may provide sufficiently good results. However, to capture alterations on the bone surface, a relatively good image quality with high resolution is required, which may need to expose the tissue to doses of 2 Gy or even higher. In such cases the applied dose is not negligible and may result in potential side effects on the cellular level as we demonstrated (Figure 2, 3).

Our results indicate that the effects of ionizing radiation on survival of the pre-oesteoblastic MC3T3-E1 cells are different in our 3D model system with collagen type I matrices mimicking natural bone-like tissue as compared to 2D culture plates. We show considerably higher survival rates when similar doses are applied as previously reported for 2D studies (Figure 2A,B). For instance, it was demonstrated, that osteoblast-like cells from the calvarium of newborn rats survived only by 60 %, 35 % and less than 5 % on a 2D surface when 2, 4 and 6 Gy, respectively, were applied to the culture [5]. The reason for this difference is unclear. We speculate that the 3D organization of the cells mimicking a more natural tissue-like environment than a 2D culture may have a protective effect but also supports repair mechanisms more efficiently after irradiation damage. In addition, shielding effects of the PMMA sample holder may not be totally excluded.

Expression of ALP-activity is a relevant marker in the early differentiation process of MC3T3-E1 pre-osteoblastic cells towards mature osteoblasts as it is upregulated with proceeding maturation [18]. We show here a time-dependent decrease of ALP-activity after exposure to radiation (Fig. 3). While no significant alteration was found 2 d post-irradiation, the ALP-activity was significantly decreased at 7 d by around 60 %. We speculate that differentiation and maturation processes may be inhibited after irradiation. However, it is not clear, by which mechanisms the ALP-activity is influenced by irradiation. It may be as result of altered cytokine expression profiles, by the irradiation directly or by combined processes [6]. Other authors found inhibition of differentiation, when exposing mature osteoblasts in a 2D environment to 4 Gy doses. In their study, ALP-activity normalized to the DNA-content was shown to be decreased by around 20 % after 3 d of irradiation, around 60 % after 6 d, and around 70 % after 9 d. However, a study using 3D scaffolds prepared from silk fibroin and human mesenchymal stem cells did not demonstrate alterations in ALP-activity after repeated high-resolution μCT imaging over a period of 28 d [13]. The reasons for these controversial results on the ALP-activity may include different study designs including different cell types, radiation doses, the different times between the μCT imaging and the ALP-activity assays, which may allow DNA repair processes in some cases, as well as the different biophysical and biochemical properties of the 3D scaffolds used in the respective studies.

The direct transfer of the results obtained in this study into *in vivo* experiments may not be possible, however the *in vitro* model system applied here, allows conclusions for *in vitro* tissue engineered constructs or bone chambers (i.e. critical dose). The critical dose for *in vivo* applications needs to be assessed with the respective *in vivo* models, specifically the effects on bone tissue growth, healing and turnover. Potential immune reactions, vascularization of the

tissue, and potential shielding effects of the surrounding tissue, may affect the critical dose *in vivo.*

Conclusion

We have established a model system allowing the monitoring of 3D tissue engineered constructs using μCT imaging. Our results indicate significant impairments on the cellular level after imaging with high-resolution μCT in a temporal and dose-dependent manner. Results from this study indicate that imaging with high-resolution μCT with doses as low as 1.9 Gy, which is required to obtain a reasonable image quality, can yield irreparable damages on the cellular level. It is therefore recommended to use the lowest resolution necessary in monitoring studies of bone chambers and 3D tissue engineered bone-like constructs. Future studies may include other cells such as mesenchymal cells present in bone tissue, and/or different scaffolds, as well as longitudinal monitoring. Longitudinal animal studies may be performed to assess a critical *in vivo* dose to avoid potential damages on the cellular level.

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Author Disclosure Statement

The authors declare no competing financial interests.

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Figure Legends

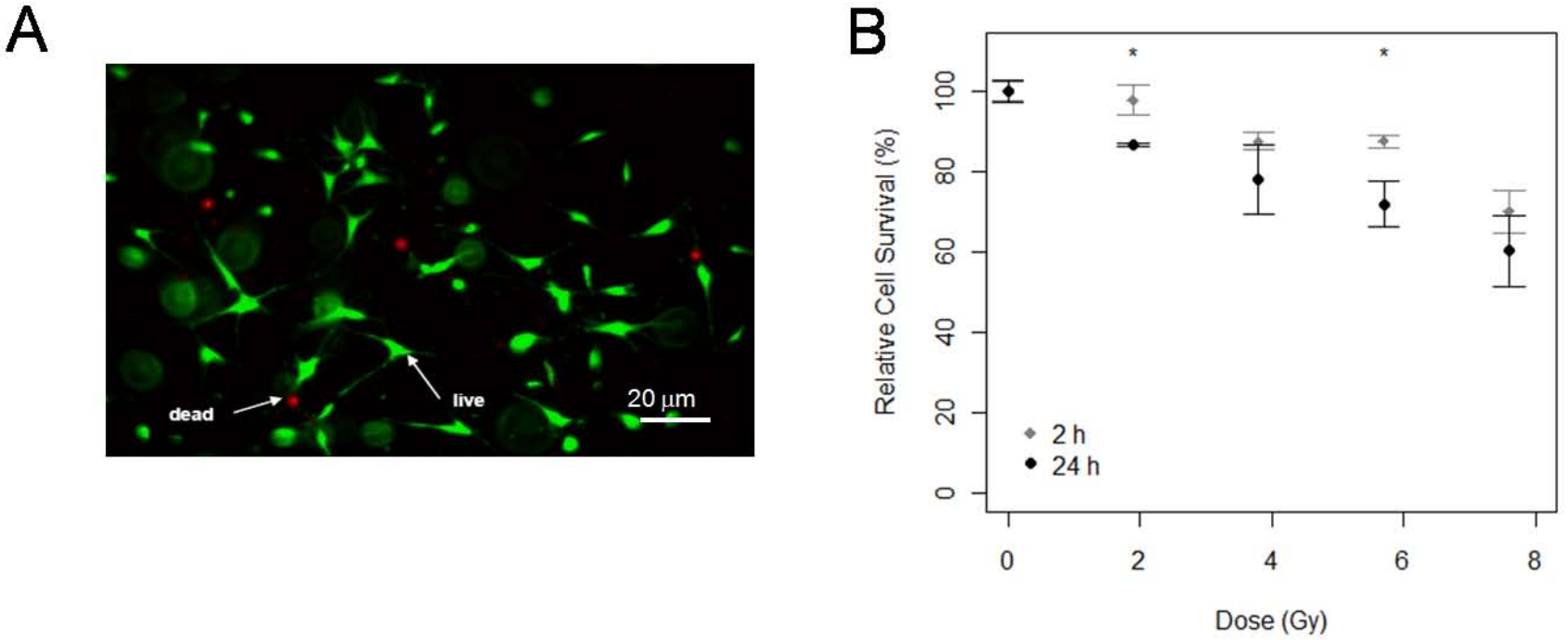
Figure 1: Relation of μCT image quality and applied dose. (A) μCT images of a bovine trabecular bone sample. Inserts show higher magnifications and the corresponding binary images, respectively. These scans were performed in four different modes; Standard resolution at 30 μm and 16 μm nominal resolution (voxel size), and High resolution at 15 μm and 8 μm nominal resolution. The nominal resolution corresponds to the scanner settings and not to the actual spatial resolution. (B) The relation between dose and image quality (here given as nominal resolution) can be modeled as a power law with exponents 1.74, 1.76, and 2.03, for Standard, Medium and High resolution mode. (C) Radiation dose in function of the imaging mode. For all cell experiments the Medium resolution mode at 20 μm nominal resolution was used, but with a threefold integration time resulting in a calculated base dose of 1.9 Gy per measurement. To achieve the higher doses of 3.8, 5.7, and 7.6 Gy, the base measurement was repeated 2, 3, and 4 times, respectively.

Figure 2: Survival of pre-osteoblastic MC3T3-E1 cells in 3D collagen matrices after μCT imaging. (A) Representative image of pre-osteoblastic cells in 3D collagen matrices 24h post-irradiation. (B) Dose and time-dependent survival 2 h and 24 h after irradiation as assessed by quantifying Live/Dead cell staining. At a dose of 2 Gy, which is commonly applied for in vitro and in vivo imaging, the fraction of surviving cells 24h after irradiation is significantly lower than at 2h. The highest dose applied (8 Gy) still resulted in survival of > 60% both at 2h and 24h after irradiation. The survival rates at each dose were normalized by the survival rates of the control samples and are shown as mean values ± standard deviation ($n_{Samples}$ = 3, $n_{Controls}$ =9; images were taken at five randomly selected/ representative locations within the matrix).

Significant differences were accepted for $p < 0.05$. * indicates significantly different data between 2 h and 24 h.

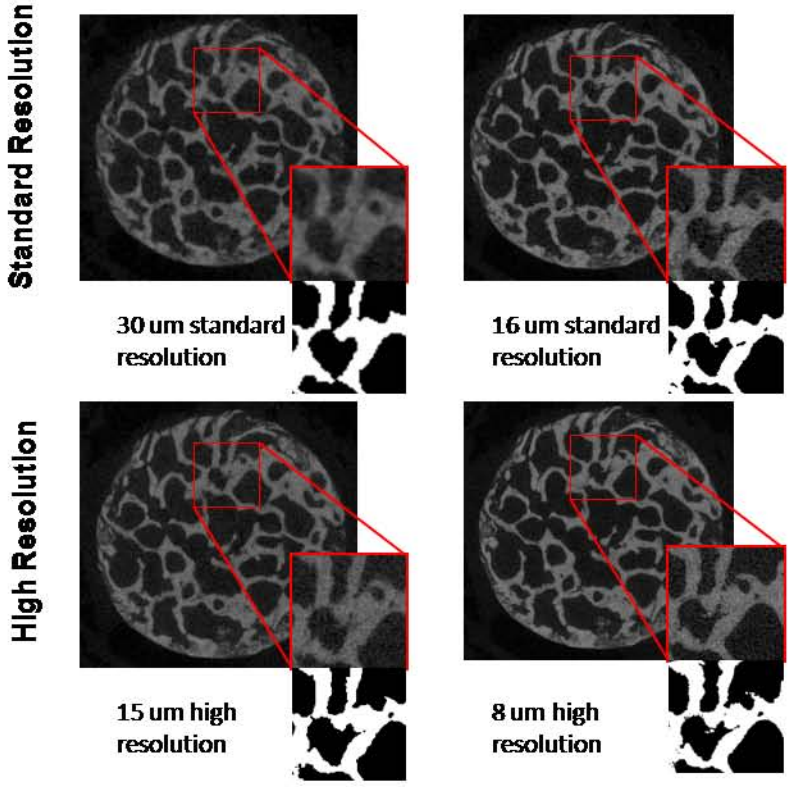
Figure 3: Time-dependent decrease of ALP-activity after exposure to 1.9 Gy radiation dose. While no significant alteration in the 2 d post-irradiation group was found, the ALP-activity was significantly decreased by 60 % at 7 d post-irradiation. In these box plots, the box represents/contains 50 % of the data, and the whiskers show the data range excluding extreme values that are shown as single data points. Differences between two data sets were considered statistically significant for $p < 0.05$ and are indicated in the figure by a star.

Figur 2

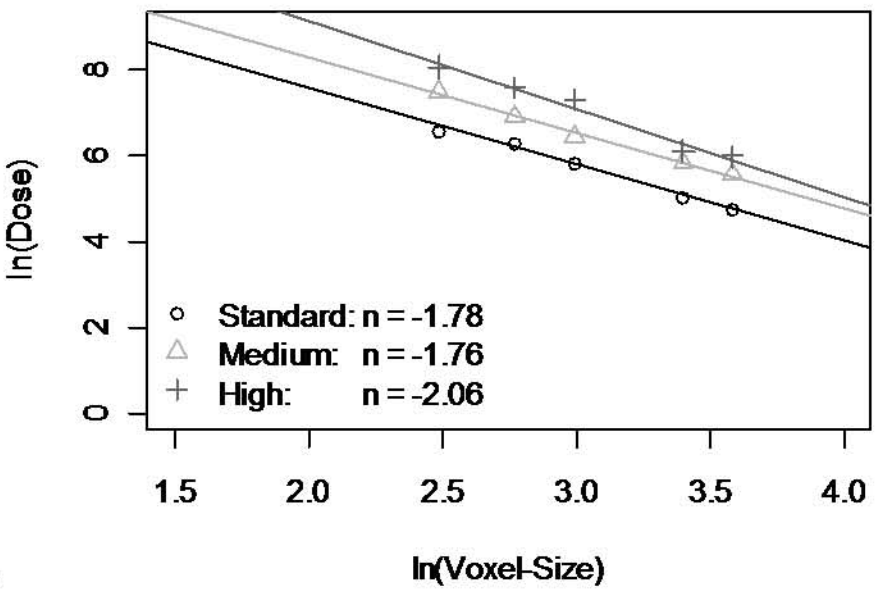

A
dead
live
20 µm
B
Relative Cell Survival (%)
0
20
40
60
80
100
2 h
24 h
*
*
0
2
4
6
8
Dose (Gy)

Figur 1

A



B



C

Nominal Resolution [µm] / Imaging Mode	36 (18)	30 (15)	20 (10)	16 (8)	12 (6)
Standard Resolution [Gy]	0.117	0.150	0.335	0.528	0.720
Medium Resolution [Gy]	0.261	0.349	0.633	1.028	1.839
High Resolution [Gy]	0.400	0.458	1.519	1.980	3.250

Figur 3